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(54) Novel receptors: their identification, characterization, preparation and use

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(56) References cited:
EP-A- 0 325 849 WO-A-88/03168

- SCIENCE, vol. 240, 13th May 1988, pages 889-895, Lancaster, PA, US; R.M. EVANS: "The steroid and thyroid hormone receptor superfamily"
- CELL, vol. 55, 2nd December 1988, pages 899-906, Cell Press, Camdridge, NA, US; S.M. HOLLENBERG et al.: "Multiple and cooperative trans-activation domains of the human glucocorticoid receptor"
- CELL, vo. 46, 29th August 1986, pages 645-652, Cell Press, Cambridge, NA, US; V. GIGUERE et al.: "Functional domains of the human glucocorticoid receptor"
- CELL, vo. 49, 10th April 1987, pages 39-46, Cell Press, Cambridge, NA, US; S.M. HOLLENBERG et al.: "Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor"
- NATURE, vol. 334, 7th July 1988, pages 31-36, London, GB; D. METZGER et al.: "The human oestrogen receptor functions in yeast"
- CELL, vol. 52, 29th January 1988, pages 161-167, Cell Press, Cambridge, NA, US; H. KAKIDANI et al.: "GAL4 activates gene expression in mammalian cells"

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Description

The present invention relates to novel receptors, their identification, characterization, preparation and use.

Reference is made to Patent Cooperation Treaty International Application Publication No. WO 88/03168 and European Patent Application Publication No. 0,325,849. These published applications refer in various respects to hormone receptors and compositions thereof, and to methods for their preparation, and use, particularly in novel assay systems.

The present invention relates generally to the identification and characterization of certain polypeptide sequences that function as transcription trans-activation domains, and to their preparation and use, particularly in the preparation of novel intra-cellular hormone or hormone-like receptors, for example, steroid receptor polypeptides, thyroid polypeptides and retinoid polypeptides including those of the human species, where advantage is provided in terms of trans-activation transcription initiation activity by augmenting the effect of said domains.

More particularly, the present invention is directed to such novel receptor polypeptides, wherein the transcription trans-activation domains have been augmented in effect so as to produce novel entities that exhibit increased transcription initiation activity surprisingly superior to the parent molecule.

Novel aspects relating to the preparation of such transcription trans-activation molecules, including novel DNA isolates encoding same and the transcription trans-activation domains, expression vectors operatively harboring these DNA sequences and hosts transfected with said vectors are included within the scope of this invention.

Most particularly, the present invention concerns the use of the novel transcription trans-activation hormone or hormone-like receptors of the present invention in assays for screening various putative materials that may have operative binding affinity for the novel hormone or hormone-like receptors hereof. In a preferred embodiment, this aspect of the invention provides an assay for screening such putative materials, for example, steroid agonists and antagonists in an enhanced, so-called trans-activation system.

The patent applications cited supra disclose, inter alia., the characterization and preparation of various hormone and hormone-like receptors, including steroid, thyroid and retinoid receptors such as those represented by the glucocorticoid, mineralcorticoid, thyroid, estrogen related and retinoid classes, and specifically, the glucocorticoid, estrogen, aldosterone and retinoic acid receptors themselves. These specific receptors have been the subject of considerable research and form the particular bases for the inventions disclosed and claimed in these patent applications. Similarly, the extant, parallel scientific literature has focused on the specific receptors listed above from among the classes of receptors that exist.

It is known, for example, that the glucocorticoid receptor belongs to a large super-family of ligand-dependent transcription factors that have themselves diverse roles in homeostasis, growth and development. Comparison of complementary DNAs encoding these receptors, as well as mutational analyses of their coding sequences have identified certain functional domains within the molecule that are thought responsible respectively for DNA binding, hormone binding and nuclear localization. See Evans, et al., Science 240, 889 (1988) for a review of this subject matter. In the case of the glucocorticoid receptor, the so-called DNA binding domain spans some sixty-six amino acids and is highly conserved among various species and this domain has been found to be required in order to activate transcription. See Hollenberg, et al., Cell 49, 39 (1987), Miesfeld, et al., Science 236, 423 (1987), Danielsen, et al., Mol.Endo 1, 816 (1987), Kumar, et al., Cell 51, 941 (1987), Gronemeyer, EMBO J. 6, 3985 (1987), and Waterman, et al., Mol.Endo 2, 14 (1988). This region has been found to contain nine invariant cysteines residues and although the contribution of each cysteine residue to overall function is unknown, as is the actual structure formed by this domain, it has been proposed that these cysteine residues coordinate two zinc ions to form two DNA binding, so-called finger domains which result in a ternary structure thought responsible for its localization and binding to the requisite DNA site. See Klug, et al., Tr.Biochem.Sci 12, 464 (1987), Bens, et al., Cell 52, 1 (1988), and Evans, supra.

In a location nearer the carboxy-terminal end distal from the DNA binding region is the so-called ligand binding domain which has the demonstrated ability to block activity of the receptor in the absence of hormone. Thus, presence of the requisite hormone relieves the inhibition of the receptor to activity. Deletion of this region has been found to produce a hormone-independent transcription activator. See Godowski, et al., Nature 325, 365 (1987), Hollenberg, et al., supra, Kumar, et al., supra, Danielsen et al., supra, and Adler et al., Cell 52, 685 (1988).

In contrast to these two domains, the sequences lying towards the amino-terminal region from the DNA binding domain is poorly understood both as to structure, and particularly, function. This region is extremely variable both in size and in composition amongst the various receptors - See Evans, supra - and may contribute to the heterogeneity of receptor function. See Kumar et al., supra, and Tora et al., 333, 185 (1988).

Despite extensive analysis, some of which having been reported in the scientific literature, the region(s) that determines trans-activation of transcription initiation remains poorly characterized. Trans-activation domains can be defined as polypeptide regions that, when combined with the DNA binding functional domain, increase productive transcription initiation by RNA polymerases. See Sigler, Nature 333, 210 (1988), Brent et al., Cell 43, 729 (1985), Hope et al., Cell 46, 885 (1986), Ma et al., Cell 48, 847 (1987), Ma et al., Cell 51, 113 (1987), Lech et al., Cell 52, 179 (1988), and Hope et al., Nature 333, 635 (1988).

Previous research of the human glucocorticoid receptor by linker scanning mutagenesis identified two regions outside of the DNA binding region having a role in transcription activation. These regions were defined as τ_1 and τ_2. Giguere et al., Cell 46, 645 (1986). Further research from these laboratories has also resulted in the report of a co-localization of trans-activation and DNA binding functions. See Hollenberg et al., supra, Miesfeld, et al., supra, Danielsen et al., supra, and Waterman et al., supra. As a composite, this research has given rise merely to an emerging picture of an increasingly modular molecule with discrete domains, each contributing to the identified properties of ligand-binding, DNA-binding and trans-activation of transcription. However, until now, the region(s) determining the trans-activation activity, was not at all well understood. Thus, the picture based upon existing research lacks an appreciation of the dynamic nature of the steroid receptors and how the various domains determine the cascade of events initiated by ligand-binding and consummated by promoter-specific trans-activation.

Further, although previous research has identified functional "domains", there has been little systematic effort to identify amino acids that contribute to the specific activities of the molecule itself. Thus, the previous identification of steroid receptor trans-activation regions resulted only from a demonstrated loss of activity via deletion or insertional mutagenesis, but in no case have the properties of the regions themselves been confirmed in assays that reflect a dominant gain of function.

Thus, Godowski et al., Science 241, 812 (1988), report results that show that the glucocorticoid receptor contains at least one "enhancement domain" other than that overlapping the glucocorticoid response element binding region and that the second domain occupies a region near the receptor amino-terminus. Similarly, Webster et al., Cell 54, 199 (1988) report on an inducible transcription activation function of the estrogen and glucocorticoid receptors, and these researchers speculate that the relative positions of the hormone regions (i.e., ligand and DNA-binding domains) are not important for the transcription induction activity of the receptor. Yet, these researchers admit that they have no definition of the exact location and nature of what they call the hormone-inducible activating domain, to say nothing of its characterization and role in trans-activating potential.

As a starting point for the present invention, Giguere et al., supra, demonstrated loss of activity in the glucocorticoid receptor based upon an assay measuring transcription activity, when random site-directed mutagenesis was performed at several locations of the molecule. As a followup, Hollenberg et al. deleted regions within the molecule, again demonstrating overall loss of transcription activity induced by such removal of stretches of amino acids.

It is an object of the present invention to identify and characterize the domain(s) responsible for trans-activation transcription activity, and the characterization of such domain(s) in respect of amino acid composition and sequence, to explore the functional interaction of the domain(s), if any, with both the DNA-binding and ligand-binding domains of a given receptor, and finally, to exploit such knowledge via the manipulation of such identified and characterized trans-activation transcription domain(s) so as to increase the overall transcription activity of the given receptor so manipulated.

The present invention thus provides novel hormone or hormone-like receptors that have been modified by advantage of knowledge of the identity and characterization of the trans-activation transcription activity domain(s), by modifications thereof so as to produce novel, heterologous receptors that have increased activity compared with the parent molecule. It is an object of the present invention to provide novel, heterologous, optionally hybrid receptors having increased trans-activation transcription activity and otherwise having DNA-binding and ligand-binding domains that may be borrowed from various different receptors. It is a further object of the present invention to provide novel assays whereby putative receptor agonists and antagonists can be screened and evaluated for potential commercial exploitation. See also Ptashne, Nature 335, 683 (1988).

Summary of the Invention

The present invention is predicated upon the identification, isolation and characterization of the trans-activation transcription domains of intracellular hormone or hormone-like receptor polypeptides that has in turn enabled the discriminate characterization of the receptor itself, both in terms of physical attributes and the biological function and effect of their various domains. This information has in turn enabled the production of harnessed, recombinant systems useful for preparing the novel receptors hereof having augmented transcription activation properties.

It has been determined, based upon the information provided herein, that receptors contain trans-activation transcription domains that are position independent and autonomous in function. Thus, the present invention provides for novel hormone or hormone-like receptors wherein the trans-activation transcription domains are augmented in their ability to activate transcription. Such novel receptors of this invention contain trans-activation transcription domains additional to the parent molecule, positioned in a manner to provide further increase in transcription activity. These novel receptors may be hybrids wherein the DNA-binding and the ligand-binding domains are provided from receptors of the same or different class and/or species.

The present invention is also directed to the use of such novel receptors for in vitro bio-assays for determining the functionality of a putative receptor or a putative hormone or hormone-like material. Bio-assays may take the form, for

example, of challenging a novel receptor hereof with one or more of a battery of test materials that have putative hormone or hormone-like activity and that can potentially modulate the bio-function of said receptor and monitoring the effect of said material on said receptor in an in vitro setting.

The present invention is further directed to the preparation of such novel receptors hereof via recombinant DNA technology in all relevant aspects, including a DNA molecule that is a recombinant DNA molecule or a cDNA molecule consisting of a sequence encoding said receptor or a trans-activation transcription domain thereof, and to requisite expression vectors operatively harboring such DNA comprising expression control elements operative in the recombinant host selected for the expression, and to recombinant host cells transfected with such operative expression vectors.

The present invention is further directed to a method for inducing the expression of DNA encoding a reporter molecule or other desired heterologous polypeptide comprising inducing transcription of the DNA encoding said polypeptide by a complex formed by a novel receptor hereof and a corresponding ligand capable of binding to said receptor, in an in vitro setting wherein said receptor and said DNA encoding said polypeptide are produced via recombinant expression in a transfected cell host system.

The present invention thus embraces a hormone or hormone-like receptor as a polypeptide having increased trans-activation transcription activity of a promoter with which it is associated, by virtue of its intrinsic ability to bind to a DNA sequence response element of said promoter or by its ability to associate with other polypeptide(s) that bind to said DNA sequence response element, and having trans-activation transcription activity greater than that of its corresponding parent receptor.

The present invention is directed to recombinant DNA technology in all aspects relating to the use of the characterization of the trans-activation transcription domain of a hormone or hormone-like receptor for DNA isolates production, including cross-hybridizable DNA isolates, devising expression vectors therefor, transfected hosts: produced therewith and methods comprising a method of use utilizing such information to devise cells or cell lines harboring genetic information sufficient for such cells or cell lines to produce such receptors such that they can be used as such or in expression systems or in assays for determining the activity of corresponding putative ligands.

Detailed Description of the Invention

1. Brief Description of the Drawings

Figure 1 depicts the identity, and transcription activity, of various human glucocorticoid receptor (hGR) entities hereof that have been modified from the parent molecule in the trans-activation transcription domains (tau_1). Wild-type hGR (wt) and tau1 mutants are schematically represented. Functional regions are hatched (tau_1), stippled (DNA-binding domain), or indicated by "DEX" (hormone binding domain). Numbers above each receptor define amino acid positions. Heavy vertical bars identify boundaries of an inserted fragment. Relative luciferase (reporter molecule) activity was measured by MTV-LUC using 10^{-7} M dexamethasone (corresponding steroid), except receptors indicated by "C" after the activity which are constitutively active.

Figure 2 sets forth the luciferase activity of various tau_2 receptors hereof. The wild-type hGR is represented at the top. The tau_2 region extends from amino acids 526 to 556 and is represented by a solid rectangle. Replacements of the tau_1 region are indicated by a solid rectangle (tau_2) or by hatched rectangles for the amphipathic alpha helix ("aah"). Relative luciferase activities were measured by MTV-LUC in the presence of 10^{-7} dexamethasone and are followed by "(C)" when hormone-independent. Asterisks indicate site of the amino acid end of truncated molecule.

Figure 3 depicts the construction of hybrid steroidal receptor of glucocorticoid-thyroid hormone receptors whose trans-activation transcription activity is increased by the addition of tau_1 domains. A segment of the human glucocorticoid receptor cDNA encoding amino acid 77 to 262 (encoding the tau_1 domain) was inserted in the rat alpha thyroid hormone receptor cDNA at a position corresponding to amino acid 21, in one or multiple copies. The parental receptor is rTR alpha with a BamHI linker inserted at the unique BstEII site in the amino terminus. Constructs were transfected into CV-1 cells with TRE-CAT and CAT activity measured in the absence or presence of T_3.

Figure 4 sets forth the point mutational analysis of the hGR DNA-binding domain. The amino acid sequence of the hGR DNA-binding domain is given. Each line represents information believed to be encoded by part of a separate exon. The consensus sequence (con) for the steroid hormone receptor super-family is presented below the hGR sequence, with invariant (bold), conserved(standard type) and non-conserved (dashes) amino acids indicated. Amino acids converted to lysine are topped by circles. Transcription activity of mutants assayed with MTV-CAT and compared with hGR-SV are indicated as greater than 10% (filled circles), 1% to 10% (half-filled circles), and less than 1% (open circles).

2. General Methods and Definitions

Amino acid identification uses the single- and three-letter alphabets of amino acids, i.e.:

Asp	D	Aspartic acid	Ile	I	Isoleucine
Thr	T	Threonine	Leu	L	Leucine
Ser	S	Serine	Tyr	Y	Tyrosine
Glu	E	Glutamic acid	Phe	F	Phenylalanine
Pro	P	Proline	His	H	Histidine
Gly	G	Glycine	Lys	K	Lysine
Ala	A	Alanine	Arg	R	Arginine
Cys	C	Cysteine	Trp	W	Tryptophan
Val	V	Valine	Gln	Q	Glutamine
Met	M	Methionine	Asn	N	Asparagine

Steroid receptors hereof are prepared 1) having methionine as the first amino acid (present by virtue of the ATG start signal codon insertion in front of the structural gene) or 2) where the methionine is intra- or extracellularly cleaved, having its ordinarily first amino acid, or 3) together with either its signal polypeptide or conjugated protein other than its conventional signal polypeptide, the signal polypeptide or a conjugate being specifically cleavable in an intra- or extracellular environment. In all events, the thus produced receptor, in its various forms, is recovered and purified to a level suitable for intended use. See Supra.

The "hormone or hormone-like receptors" of this invention include the receptors specifically disclosed, for all species that cross-hybridization exists, most notably other mammalian receptors, as well as related (e.g., gene family) receptors of the same or cross-hybridizable species that are enabled by virtue of DNA isolation and characterization and use via cross-hybridization techniques from said specific receptors or from identification via immuno cross-reactivity to antibodies raised to determinants in the usual manner known per se. It also includes functional equivalents of all of the above, including interspecies or intraspecies receptors wherein DNA-binding and/or ligand-binding domains are swapped with one another, or otherwise differing in one or more amino acids from the corresponding parent, or in glycosylation and/or phosphorylation patterns, or in bounded conformational structure.

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, where such sequences are operatively linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors may be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. "Operative," or grammatical equivalents, means that the respective DNA sequences are operational, that is, work for their intended purposes. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified DNA sequence disposed therein is included in this term as it is applied to the specified sequence. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form, are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

Apart from the novelty of the present invention involving the manipulation by means of repositioning or augmentation of the trans-activation transcription domains of a parent steroid receptor, it will be understood that the novel steroid receptors of the present invention may otherwise permissively differ from the parent steroid receptor in respect of a difference in one or more amino acids from the parent entity, insofar as such differences do not lead to a destruction in kind of the basic steroid receptor activity or bio-functionality.

"Recombinant host cells" refers to cells which have been transfected with vectors constructed using recombinant DNA techniques.

"Extrinsic support medium" includes those known or devised media that can support the cells in a growth phase or maintain them in a viable state such that they can perform their recombinantly harnessed function. See, for example, ATCC Media Handbook, Ed. Cote et al., American Type Culture Collection, Rockville, MD (1984). A growth supporting medium for mammalian cells, for example, preferably contains a serum supplement such as fetal calf serum or other supplementing component commonly used to facilitate cell growth and division such as hydrolysates of animal meat or milk, tissue or organ extracts, macerated clots or their extracts, and so forth. Other suitable medium components include, for example, transferrin, insulin and various metals.

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and

eukaryotic organisms.

In addition to the above discussion and the various references to existing literature teachings, reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques encompassed by the present invention. See, for example, Maniatis, et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1982 and the various references cited therein, and in particular, Colowick et al., Methods in Enzymology Vol 152, Academic Press, Inc. (1987). All of the herein cited publications are by this reference hereby expressly incorporated herein.

The foregoing description and following experimental details set forth the methodology employed initially by the present researchers in identifying and characterizing and preparing particular receptors. The art skilled will recognize that by supplying the present information including the location and makeup of the trans-activation transcriptional domain of a given receptor and how it can be manipulated to produce the novel receptors hereof, it is not necessary, or perhaps even scientifically advisable, to repeat these details in their endeavors to reproduce this work. Instead, they may choose to employ alternative, reliable and known methods, for example, they may synthesize the underlying DNA sequences encoding a particular novel receptor hereof for deployment within similar or other suitable, operative expression vectors and culture systems. Thus, in addition to supplying details actually employed, the present disclosure serves to enable reproduction of the specific receptors disclosed and others, and fragments thereof, using means within the skill of the art having benefit of the present disclosure. All of such means are included within the enablement and scope of the present invention.

3. Detailed Description of Particularly Preferred Embodiments

The present invention was premised upon use of the glucocorticoid receptor as a model herein for the preparation of novel modified entities thereof including hybrids of the glucocorticoid receptor with other receptors such as the thyroid receptor, particularly in the swapping of DNA-binding and ligand-binding domains to make up such hybrids. In each case the essence of this invention, namely, the repositioning and/or augmentation of the trans-activation transcription domain(s) so as to create novel receptors whose trans-activation transcriptional activity is increased over the parent, is illustrated herein using the glucocorticoid receptor as the parent or hybrids thereof upon which comparisons were made for the novel trans-activation transcription domain modified versions.

It will be understood therefore that for receptors that are known in the art, whether wild-type, hybrids, or functional equivalents as set forth herein, they are suitable as starting materials for the trans-activation transcriptional domain(s) modifying aspects of the present invention.

4. Examples

The following examples detail materials and methods employed in the experimental procedures that follow:

Point Mutagenesis and Transcriptional Activation

To define systematically the role of the DNA binding domain in the function of the receptor, research employed extensive site-directed mutagenesis of this conserved region. By testing the role of individual amino acids this method has the potential to determine whether mutants which affect trans-activation are independent from those which influence DNA binding.

The sequence of the hGR DNA binding region is given in Figure 4, followed by the consensus sequence for the steroid hormone receptor superfamily. Among members of the superfamily, this domain contains 20 invariant and 12 conserved residues. All invariant amino acids, as well as eight conserved and eight non-conserved residues, were changed to glycine. The ability to stimulate transcription from the GR-responsive MTV promoter and to complex specifically with a glucocorticoid response element (GRE) DNA fragment in vitro was measured.

The steroid-dependent enhancement of transcription from MTV-CAT for each mutant is given in Table I and is indicated above each altered amino acid in Figure 4. All activities are relative to the wild type receptor hGR-SB, which yields an average induction of approximately 1000 fold. Thus, even 1% residual activity is significant. The deletion mutants listed in Table I, delta420-451 and delta450-487, remove the first and second halves of the DNA binding domain, respectively, with the break point corresponding to an exon-exon boundary within the human mineralocorticoid and chicken progesterone receptor cDNAs. Both have no measurable activity indicating that neither "finger" alone is sufficient for activation.

Of the glycine point mutants, all of those which alter one of the nine invariant cysteine residues destroy activity, consistent with the idea that they are critical for function. An additional, non-conserved cysteine at position 431 is not necessary for function. Surprisingly, only five of the remaining 24 glycine mutants are completely inactive. The five non-functional alleles are all invariant residues, suggesting that they are involved in general aspects of DNA binding,

not in determining sequence specificity. However, not all invariant residues are critical for function. For example, when aspartic acid 426 is converted to glycine there is almost no loss in activity. Seven out of eight mutants with conserved amino acids changed to glycine are functional, with half of this group retaining greater than 40% activity. Similarly, mutation of non-conserved amino acids within the DNA binding domain in no instance produces a receptor with less than 10% activity and generally causes little reduction in function. Thus, despite some significant exceptions, a good correlation exists between the conservation of an amino acid and the extent of functional loss when converted to glycine, with all invariant cysteines playing a critical role for function.

Table 1.

Comparison of Transcriptional Activity and DNA Binding for hGR Glycine Mutants		
hGR Mutant	Relative CATa Activity	Relative DNA Binding[b] in vitro
hGR-SB	100	100
Δ420-451	<1	1
Δ450-487	<1	1
G421	<1	<1
G423	<1	<1
G424	<1	1
G426	75	75
G431	80	10
G432	10	30
G433	2	1
G437	20	8
G438	<1	<1
G441	<1	<1
G442	<1	60
G444	<1	1
G445	<1	<1
G446	40	6
G447	<1	2
G450	60	50
G453	90	60
G455	80	6
G457	<1	2
G460	55	90
G462	80	40
G463	<1	1
G465	1	1
G467	40	6
G470	3	4
G471	10	6
G473	<1	<1
G474	60	95
G476	<1	<1
G477	<1	1
G479	65	40
G480	90	50
G481	<1	3
G486	10	1

a Activity was measured with MTV-CAT in CV-1 cells.

b The DNA binding assay is described elsewhere (Hollenberg et al., 1987). The value given was derived from total immunoprecipitated counts but reflects specific binding as determined by gel electrophoresis.

In vitro DNA Binding of Point Mutants

The CAT activity measured in the transcription assay is the sum of multiple individual functions including nuclear localization, DNA binding, dimerization and perhaps the allosteric events and protein-protein interactions that ultimately result in activation. If more than one essential function is encoded by the DNA binding domain, some of the non-functional point mutants may still retain their ability to bind DNA but fail to activate. To explore this possibility, each mutant protein was produced by transfection of the corresponding expression vector into COS-1 cells and assayed for its ability, in crude extracts, to form a specific DNA-protein complex with radiolabeled DNA. The activity of each mutant in this immunoprecipitation DNA binding assay is given in Table I; the value shown represents specific binding of the GRE fragment. In general, there is good correspondence between the ability to activate transcription and to bind DNA in vitro. Mutants for which DNA binding activity is significantly lower than transcriptional activity, such as G446 G455, G467 and G486, may be unstable in vitro. The transcriptional activation measured for these mutants is a result of specific interaction with DNA in vivo, since deletion of GREs from the MTV promoter abolishes their activity.

Only a single mutant, G442, which converts the lysine directly following the first putative finger to glycine, has lost the capacity to efficiently stimulate transcription while maintaining affinity for DNA in vitro. The specificity of binding to GREs relative to non-specific sequences is only minimally affected, indicating that its dramatic loss in activity is not due to an inability to bind promoter sequences in vivo. This is an intriguing mutant because it suggests that DNA binding is not sufficient for activation. Perhaps G442 prevents a secondary event, such as an allosteric change, following the primary protein-DNA interaction. Despite this exception, these results demonstrate the cysteine-rich 66 amino acid region to embody the DNA binding domain. Indeed, although DNA binding is necessary for trans-activation this function must be located in other regions of the receptor.

The G442 species has particular significance in terms of utility because it is the single species prepared that fails to result in activity but still shows evidence of substantial DNA binding. It is contemplated, for example, that an assay can be devised exploiting the property of both G442 and the I550* species. The I550* species lacks completely a ligand-binding domain, and as such is not responsive to the presence or absence of hormone. The hormone to a specific ligand-binding site relieves the inhibition of the molecule to act as a trans-activation transcription factor. Lacking this domain means that the I550* species will always produce activity in an assay with or without presence of steroid. On the other hand, the G442 species in the same assay will always be inactive with or without steroid. By devising an appropriate assay where both receptors G442 and I550* are present in the system, initially there will be 100% activity based upon the contribution of the I550* species alone. As the appropriate steroid is added to the system, the activity observed will fall increasingly toward zero with time. Administration of a putative antagonist along with the appropriate steroid, if active as such, would restore the activity as the antagonist would interfere with the action of the steroid thus reducing the overall activity and a rebound in activity would be seen.

GAL4/hGR Chimeras

If DNA binding and τ functions are truly separable, the possibility exists to replace the hGR DNA binding domain with a non-receptor DNA binding domain to produce a hybrid activator protein with a new promoter specificity. To test this possibility, the hGR cysteine-rich region was substituted with the first 74 amino acids of yeast GAL4. These GAL4 amino acids are sufficient for sequence-specific DNA recognition, but have no transcriptional activation capability. The ability to trans-activate lies outside the DNA binding domain and is encoded in two separate regions of the protein. Therefore, in order to produce a functional transcription factor fusions between the GAL4 DNA binding domain and hGR must contain trans-activation functions contributed by the hGR.

To assay function of hGR GAL4 hybrids, a GAL4-responsive promoter, deltaMTV-GAL-CAT, was constructed from MTV-CAT. This fusion gene was rendered GR-non-responsive and GAL4-inducible by replacement of GRE sequences with a synthetic GAL4 binding site. When measuring the activation of hGR and GAL4 on this promoter, hGR cannot produce measurable stimulation from this promoter, whereas GAL4 can increase CAT expression about 20-fold relative to a control expression vector. This is consistent with previous reports that GAL4 can function in higher eukaryotic cells. The stimulation by GAL4 is clearly mediated through the GAL4 synthetic binding site; deletion of this site renders the promoter non-responsive to GAL4.

Fusions between the GAL4 DNA binding domain and potential hGR activation domains were examined for their ability to stimulate the GAL4-responsive reporter. To demonstrate that the hGR DNA binding domain is not required for the activity of this hybrid, the GAL4 DNA binding domain was substituted for the hGR DNA binding domain to produce the hybrid GgalG. (The hGR, including amino terminus (G-), DNA binding domain (G), and carboxy terminus (G), is referred to as G-G-G, respectively. Replacement of the hGR DNA binding domain with that of GAL4 generates G-gal-G). The resultant hormone-dependent hybrid clearly can function in the absence of the hGR DNA binding domain and actually acts as a more potent transcription factor than GAL4 in this assay system, giving a 500 fold increase in

CAT activity with addition of hormone. Unexpectedly, GgalG can also stimulate delta MTV-CAT without a GAL4 binding site, but only about 5% of that measured on delta MTV-GAL-CAT. The plasmid delta MTV-AT may contain a cryptic GAL4 recognition site that is revealed only with the stronger GgalG activator and not the weaker GAL4. Indeed, activation is dependent on the GAL4 DNA binding domain; GgalG is functional on deltaMTV-CAT whereas GGG is not.

The trans-activation capability of GgalG must be determined by the amino- or carboxy-terminal regions of hGR, since the GAL4 DNA binding domain alone is inactive. Accordingly, each of these hGR regions was individually tested for its ability to complement the GAL4 DNA binding function. Both hybrids are functional with Ggal(delta) displaying constitutive activity while (delta)galG is fully hormone dependent. Therefore, autonomous trans-activation functions are embodied in both the N-terminal and C-terminal segments of the hGR, although subject to different constraints.

Rearranged and Partially Duplicated hGR Mutants

Fusion with the GAL4 DNA binding domain demonstrated the presence of distinct trans-activation properties in the hGR amino-terminal 420 amino acids and carboxy-terminal 300 amino acids. Mutants of the hGR with tau_1 duplications were assayed on the luciferase derivative of MTV-CAT, MTV-LUC. Activity values determined with the MTV-luciferase fusion gene and MTV-CAT are equivalent for previously described deletion mutants. Figure 1 shows the series of τ_1 mutant derivatives and their luciferase activity relative to wild type receptor. Absence of τ_1 reduces activity to 5%, while the tandem duplication mutant GR11 acts as a "super" receptor with 310% activity. Mutants GR12, GR14, GR17 and GR18 reveal that τ_1 can function between the DNA and hormone binding regions, as well as on the amino terminal side of the DNA binding domain, giving rise in each case to a hormone-dependent activator (Figure 1). This indicates a remarkable flexibility of receptor structure. The ability of τ_1 to increase activity is independent of both the amino and carboxy termini, as shown in Figure 1 by comparison of GR13 and GR14 activities, and GR15 with I515* and GR16. The τ_1 region may not account for all trans-activation ability in the amino terminus as shown by the 4-fold greater activity of GR18 relative to GR14. Also in support of this proposition, deletion of both τ_1 and the carboxy terminus in GR15 leaves 1% residual activity, a ten-fold induction, which can be abolished by deletion of the majority of the amino terminus (compare GR15 and GR26, Figure 2).

A second region with potential trans-activation character is τ_2, located at the very amino terminal end of the hormone binding domain (amino acids 526-556). This region has five negatively charged residues in a stretch of 18 amino acids and is implicated in receptor activity by the two-fold difference in activation of truncation mutants I550* and I515* (Figure 3). To determine whether τ_2 constitutes an activator domain it was introduced adjacent to, or in place of τ_1. Figure 3 shows that this region acts to give a 3-fold increase in activity when introduced into the amino terminus independent of τ_1 (compare GR20 with "wt", GR21 with Δ77-262). A second copy gives a further 2-fold increase, so that a pair of τ_2 regions gives an overall increase in activity of six-fold (GR22). Therefore, like τ_1, the position of τ_2 in the receptor is flexible, its activity is cumulative and its function can be constitutive (e.g. GR25).

Constructions similar to the τ_2 mutants GR21 and GR22 were constructed using the synthetic amphipathic α helix, "aah," containing 20% acidic residues and demonstrated to possess trans-activation properties in the context of yeast GAL4 (Giniger et al., Nature 330, 670(1987). The size and charge characteristics of the "aah" sequence are similar to the τ_2 region, which led us to explore its potential activity in the context of the hGR. Indeed, a similar increase in activity of mutants with single or multiple copies of τ_2 and aah is observed (Figure 3; compare GR21 with GR23, GR22 with GR24), suggesting that these regions may perform equivalent functions. These results support and extend the notion of the modular nature of trans-activation domains.

Trans-activation (τ) Domains

We have defined discrete hGR trans-activation regions according to the following two criteria: deletion decreases activity and duplication increases it. By these standards two regions of the receptor, of 200 and 30 amino acids, encode trans-activation functions. The localization of these two regions does not exclude a role for additional activator sequences within the hGR. Examination of the primary sequences of τ_1 and τ_2 fails to reveal any obvious homology with the exception that both regions have acidic character. This property is noteworthy because activation domains in the yeast transcription factors GAL4 and GCN4, although lacking obvious sequence identity, are rich in acidic residues. This apparent similarity does not demonstrate that any of the identified activator regions in either GAL4, GCN4, or the glucocorticoid receptor are functioning through a common mechanism, although this seems likely. The potential for a common mechanism is further supported by the observation that the synthetic amphipathic α helix ("aah") sequence can functionally replace τ_2 and to some extent τ_1. The lack of obvious sequence and size relatedness of τ_1, and τ_2 and the yeast activation sequences leads to the view that trans-activation functions might be embodied by the net context of negatively-charged residues on the surface of the DNA-bound protein.

hGR Modularity

The modular nature of the hGR has emerged not only from primary sequence comparisons within the steroid receptor superfamily, but also by the ability to exchange functional domains to create novel chimeric activators. The DNA-binding domain of the hGR has been replaced with that of the human estrogen receptor (Green et al., Nature 325,75 and Chambon, (1987) and with the unrelated DNA-binding domain from GAL4. Its position is not critical since it can function at the amino terminus. In addition, the DNA-binding domain can be placed amino or carboxy-terminal to both τ_1 and τ_2 without compromising function of these domains. Consistent with modular properties, the position of τ_1 and τ_2 is not critical and their multimerization leads to increased receptor function. Thus, we have been able to generate receptors with activity of up to four times that of the wild-type receptor or with altered DNA binding specificity. Hybrid receptors are still hormonally inducible indicating a non-specific mechanism whereby the hormone binding domain imposes a ligand-dependent effect on the rest of the molecule. Experimental detail and discussion follows:

Plasmid and Point Mutant Construction

Plasmid hGR-SB was generated by recombination at the ClaI site between linker scanning mutants I532 and I403S (Giguere et al., Cell 46, 645 (1986)). I403S was derived from I403 by introduction of the SstI adaptor 5′ GATC-GAGCTCGC 3′ into the BamHI site. This hGR derivative is parent to all point mutants and is indistinguishable from wild-type receptor in DNA binding and transcriptonal activation. To convert the desired codon of hGR-SB to one encoding glycine, the SstI/BamHI, 400-nucleotide fragment from plasmid hGR-SB was first introduced into M13mp18 to yield single-stranded template. Synthetic oligonucleotides of 13-15 bases were then used to change the desired codon to GGN by standard techniques followed by re-introduction of the altered SstI/BamHI fragment into hGR-SB. Deletions Δ420-451 and Δ450-487 were generated with longer oligonucleotides (20mers); amino acid positions given define non-deleted residues at the deletion junction. Mutant sequences were directly determined from double-stranded plasmid DNA using alkaline denaturation (Hattori et al., Anal.Biochem 152, 232 (1986)) followed by chain termination of synthetic hGR primers.

Reporter plasmid MTV-CAT and its deletion derivative ΔMTV-CAT were generated as follows. The HindIII site of pBLCAT2 (Luckow et al.,Nucl.Acids Res. 15, 5490 (1987)) was first destroyed to generate pTKCAT-H. The HSV-thymidine-kinase promoter of pTKCAT-H was then excised by digestion with BamHI/BglII and replaced with the BamHI, MTV-LTR fragment of pMTV-TK (Kuhnel et al., J.Mol.Biol. 190, 367 (1986)), pLS-190/-181 or pLS-96/-88 (Buetti et al., J.Mol.Biol. 190, 379 (1986)) to generate MTV-CAT, -190/-181 MTV-CAT, or -96/-88 MTV-CAT, respectively. ΔMTV-CAT was constructed from -190/-181- and -90/-88 MTV-CAT by recombination between the HindIII sites of these mutants. ΔMTV-CAT was converted to MTV-GAL-CAT by introduction of the synthetic GAL4 binding site "17MX" (Webster et al., Cell 52, 169 (1988)) into the unique HindIII site. Plasmid MTV-LUC was constructed by conversion of the HindIII site of pSVOA/L-A Δ5′ (de Wet et al., Mol.Cell.Biol. 7, 725 (1987)) to XhoI and introduction of luciferase coding and SV40 polyadenylation sequences from this derivative (generated by BamHI digestion, Klenow polymerase I end-filling and XhoI digestion) into XhoI/SmaI-digested MTV-CAT.

GgalG was derived from pG525 (Laughon et al., Mol.Cell.Biol. 4, 260(1984)). Primer-directed mutagenesis was used to introduce NotI and XhoI sites at coding-sequence nucleotides -10 to -3 and +223 to +228, respectively. The GAL4 DNA binding domain was excised from this derivative by digestion with NotI and inserted into $pRShGR_{NX}$ (Giguere et al., Nature 330, 624(1987)) in place of the endogenous DNA binding domain. GgalΔ and ΔgalΔ were produced by digestion of GgalG and ΔgalG, respectively, with XhoI, followed by end-filling and ligation. ΔgalG was constructed by introduction of a synthetic oligonucleotide duplex (ΔAN, 5′ GTACCACCATGGGGC 3′) containing a consensus ribosome binding site (Kozak, Nucl.Acid.Res. 12, 857 (1984)), in place of the amino-terminal-coding, Asp718/NotI fragment of GgalG.

Generation of mutants Δ77-262, I515* and Δ9-385 has been described by Hollenberg et al., Cell 49, 39, (1987). τ_1 mutants were constructed using the BglII/BamHI fragment from I262 (Giguere et al., Cell 46, 645 (1986)), encoding amino acids 77-262 of the hGR. This fragment was inserted into the BamHI sites of hGR linker scanning mutants I262 and I515 to generate GR11 and GR12, respectively. GR13 was derived from $pRShGR_{NX}$ by replacement of the amino terminal coding sequences with the Δ AN adaptor described above. GR17 was created by insertion of the amino terminal coding BamHI fragment, generated by recombination between linker scanning mutants I9 and I384 (Giguere et al., supra,) into the BamHI site of I515. Mutant I550* was produced by recombination between the BamHI sites of mutants I550 and I696, thus shifting the reading frame after amino acid 550. This mutant incorrectly described as δ532-697 in a previous report (Hollenberg et al., supra). To create τ_2 derivatives, an exciseable τ_2 coding-fragment was generated by conversion of hGR nucleotides 1704-1709 and 1800-1805 (Hollenberg et al., Nature 318, 635 (1985)) to BamHI and BGIII sites, respectively, using oligonucleotide-directed mutagenesis. The sequence encoding τ_2 was then introduced into the BamHI site of I262 to produce GR20 or in place of the BglII/BamHI fragment of I262 to generate GR21. GR23 was created by replacement of the BglII/BamHI fragment of I262 with a synthetic oligonucleotide duplex

(5'GATCT GGAAT TACAA GAGCT GCAGG AACTA CAAGC ATTGT TACAA CAGCA AGAG 3') encoding the "aah" sequence (Giniger and Ptashne, 1987). Mutants with tandem copies of this sequence and τ_2 (GR24 and GR22) were generated by standard techniques (Rosenfeld and Kelly, 1986). Double mutant derivatives of constructions described above were generated by recombination at the ClaI site: GR14 from GR12 and GR13; GR15 from Δ 77-262 and I515*; GR16 from GR11 and I515*; GR18 from GR13 and GR17; GR25 from GR22 and I515*; GR26 from Δ 9-385 and I515*.

Immunoprecipitation DNA Binding

DNA binding was measured as described previously (Hollenberg et al., supra). Mutant receptor, obtained in a crude COS-1 cell extract after transfection, was incubated with a mixture of radiolabeled DNA fragments, one of which contained GREs. Receptor-DNA complexes were immunoprecipitated with receptor-specific antiserum and Staph A, freed of protein, counted Cerenkov, and then electrophoresed through a denaturing polyacrymide gel to verify specific binding. Total immunoprecipitated counts were compared. The presence of mutant hGR protein in each COS-1 cell extract was confirmed by Western blot analysis.

Transfection and Luciferase Assays

Transfection of CV-1 and COS-1 cells was as described previously (Giguere et al., and Hollenberg et al., supra) using 5 micrograms of each plasmid per 10 cm dish. Luciferase assays were performed as described (de Wet et al., supra).

Cell Culture and Transfection

Conditions for growth and transfection of CV-1 (African green monkey kidney) cells were as previously described (Giguere et al., Cell 46, 645 (1986)), except that the calcium phosphate precipitate was left on the cells for 4-8 hours, at which time the media was changed to DMEM with 5% T_3 free bovine serum minus or plus 10^{-7} M T_3 (Sigma). Cells were harvested 36 hours after the addition of T_3, and CAT assays were performed as described (Gorman et al., Mol. Cell.Biol. 2, 1044 (1982); Hollenberg et al., Cell 49, 39 (1987)). Typically, 5 μg reporter and 1 μg expression vector were cotransfected, along with 2.5 μg RSV-βgal as a control for transfection efficiency. Acetylated and non-acetylated forms of [^{14}C]chloramphenicol were separated by thin layer chromatography, excised, and quantitated by liquid scintillation counting in Econofluor (DuPont) with 5% DMSO. β-galactosidase assays were performed as described (Herbomel et al., Cell 39, 653 (1984)). CAT activity is expressed as percent conversion divided by β-galactosidase activity.

Construction of Reporter and Expression Plasmids

Synthetic oligonucleotides corresponding to -169 to -200 of the rat growth hormone gene or a palindromic TRE (TCAGGTCATGACCTGA) (Glass et al., Cell 54, 313 (1988)) were inserted into a linker scanning mutant of MTV-CAT that has a Hind III site at position -190/-181 (Buetti et al., J.Mol.Biol. 190, 379 (1986)) or -190/-88 MTV-CAT, which has a Hind III site replacing the nucleotides between -88 and -190. Expression vectors were constructed for the thyroid hormone receptors by inserting the full-length cDNAs of pheA12 (Weinberger et al., Nature 324, 641 (1986)) and rbeA12 (Thompson et al., Science 237, 1610 (1987)) between the KpnI and BamHI sites of the pRS vector (Giguere et al., Cell 46, 645 (1986) and Nature 330, 124 (1987)).

Construction of Chimeric Receptors.

The construction of hGR_{NX} has been described (Giguere, Nature supra). To construct $hTR\beta_{NX}$, the cDNA insert of phe A12 (Weinberger, Nature, supra) was subcloned between the KpnI and BamHI sites of M13mp19 and mutagenized by the method of Kunkel, PNAS 82, 488 (1985). The oligonucleotide used to create the NotI site changed three amino acids: Asp97 to Arg, Lys98 to Pro, Asp99 to Pro. The oligonucleotide used to create the XhoI site changed two amino acids: Thr171 to Leu, Asp172 to Gly. The mutant receptor cDNA was then transferred to the expression vector pRS (Giguere, Cell, Nature supra); hybrids were constructed by exchanging KpnI-NotI, KpnI-XhoI or NotI-XhoI restriction fragments between $RShGR_{NX}$ and $RShTR\beta_{NX}$. $RShGr_{NX}$ has about 75% of wild-type activity, and $RShTR_{NX}$ has about 60% of wild-type activity. For the addition of τ_1 to rTRα, the unique BstEII site at amino acid 21 was changed to a BamHI site by inserting an oligonucleotide adaptor that encoded a BamHI site flanked by BstEII ends. This allowed the in frame insertion of a BamHI-BglII fragment encoding amino acids 77-262 of the hGR into this site. ΔTT and ΔGG were constructed by deleting the Asp718-NotI fragment of $RShTR_{NX}$ and $RShGR_{NX}$ respectively and replacing it with an oligonucleotide adaptor of a consensus ribosome binding site (Kozak Nucl.Acids Res. 12, 857 (1984)).

Amino acids 77 to 262 of the hGR, called τ_1, were inserted in frame after amino acid 21 of rTRα in one or multiple

copies and the resulting hybrid receptors assayed for trans-activation. Table 1 shows that addition of one τ_1 domain increased activity by at least four-fold, while the presence of multiple such domains further increased activity. This is consistent with the modular nature of this domain, and demonstrates that the activity of thyroid hormone receptors can be augmented by the addition of a trans-activation domain from a different receptor.

Table 1.

Activity of thyroid hormone receptor/τ_1 hybrids.		
Receptor	# of τ_1 domains	Relative CAT Activity[a]
RShGR	1	0
RSrTRα-Bm+	0	100
RSrTR-T^{1-1}	1	1430
RSrTR-T_{1-2}	2	1140
RSrTR-T_{1-3}	3	1960
RSrTR-T_{1-4}	4	820

[a]CAT activity is relative to the induced activity of RSrTR-Bm+, which is the rat alpha thyroid hormone receptor with the BstEII site at amino acid 21 changed to a BamHI site.

In certain experiments where the amount of receptor expression vector is increased from 1μg to 5μg, relative CAT activity was shown to increase several-fold.

The foregoing description details specific methods that can be employed to practice the present invention. Having detailed specific methods initially used to identify, isolate, characterize, prepare and use the receptors hereof, and a further disclosure as to specific entities, and sequences thereof, the art skilled will well enough know how to devise alternative reliable methods for arriving at the same information and for extending this information to other intraspecies and interspecies related receptors. Thus, however detailed the foregoing may appear in text, it should not be construed as limiting the overall scope hereof; rather, the ambit of the present invention is to be governed only by the lawful construction of the appended claims.

Claims

Claims for the following Contracting States : AT, BE, CH, LI, DE, FR, GB, GR, IT, LU, NL, SE

1. An assay for screening and identifying materials having a putative potential of binding to a member of the steroid, thyroid, and retinoid superfamily of hormone receptors comprising the steps of:

   a) providing a member of the steroid hormone superfamily of receptors in a form having improved trans-activation transcription activity, wherein said receptor comprises at least one trans-activation transcription domain additional to the parent molecule selected from the group comprising τ_1, τ_2, and functional fragments thereof and located outside the DNA-binding and ligand-binding domain(s) of said receptor protein(s),
   (b) challenging the form of receptor species with one or more of a battery of test materials having putative potential of binding to a hormone or hormone-like receptor,
   (c) monitoring the effect of the test material by measuring the amount of transcription induced by the receptor species, and
   (d) selecting candidates from the battery of test materials capable of having an effect on the trans-activation transcription activity of said receptor species.

2. An assay as claimed in Claim 1 further comprising the additional step following step (d), comprising:
   (e) employing the candidate in the preparation of a composition containing the candidate as an essential component, the composition being useful to impart its biofunction properties on a corresponding receptor when it is contacted with the receptor.

3. An assay as claimed in Claim 2 further comprising the additional step following step (e), comprising:
   (f) contacting the composition with biological material obtainable from a human subject.

4. A member of the steroid, thyroid and retinoid hormone superfamily of receptors as a polypeptide having increased

trans-activation transcription activity of a promoter with which it is associated, by virtue of its intrinsic ability to bind to a DNA sequence response element of the promoter or by its ability to associate with other polypeptides that bind to the DNA sequence response element, wherein the receptor contains at least one trans-activation transcription domain additional to the parent molecule selected from the group comprising τ_1, τ_2, and functional fragments thereof, located within the molecule in a location outside of its DNA-binding and ligand-binding domains.

5. A receptor as claimed in Claim 4 wherein the receptor is based on the human glucocorticoid receptor.

6. A receptor as claimed in Claim 4 wherein the trans-activation transcription domain is the τ_1 domain.

7. A receptor as claimed in Claim 6 wherein the receptor contains two of the τ_1 domains.

8. A receptor as claimed in Claim 7 wherein the second of the τ_1 domains is located adjacent to the first.

9. A receptor as claimed in Claim 7 wherein the second of the τ_1 domains is located in a C-terminal distal location from the first τ_1 sequence.

10. A receptor as claimed in any one of Claims 4 to 9 lacking a ligand-binding domain.

11. A species of a receptor as claimed in Claim 4 which is human G442 glucocorticoid receptor, wherein by point mutation a glycine has replaced the native lysine at position 442.

12. A member of the steroid, thyroid and retinoid hormone superfamily of receptors having trans-activation transcription domains additional to that of the parent receptor selected from the group comprising τ_1, τ_2, and functional fragments thereof, autonomous with both the DNA-binding and ligand-binding domains of the parent receptor, the additional trans-activation transcription domains being located in a position outside of each of the DNA-binding and ligand-binding domains, if present, and having a DNA-binding biofunctionality.

13. A DNA molecule that is a recombinant DNA molecule or a cDNA molecule encoding a receptor as claimed in Claim 12.

14. An expression vector operatively harboring DNA encoding a receptor as claimed in Claim 13.

15. A recombinant host cell transfected with an expression vector as claimed in Claim 14.

16. A cell culture comprising cells as claimed in claim 15 and an extrinsic support medium assuring the viability of the cells.

17. A process of preparing a human receptor as claimed in Claim 12 which comprises expressing in a recombinant host cell transfecting DNA encoding the receptor.

18. A process which comprises recovering and purifying a receptor as claimed in Claim 12 to a form having quality and quantity sufficient to enable its use in assays that enable measurement of extrinsically induced biofuctionality of the receptor.

19. In a bioassay for determining the functionality of a member of the steroid, thyroid and retinoid hormone superfamily of receptors, the bioassay comprising:

(a) transfecting into receptor negative cells one or more expression vectors harboring an operative hormone responsive promoter/enhancer element functionally linked to an operative reporter or other desired DNA and an operative DNA sequence encoding the receptor,
(b) culturing the transfected cells from step (a) in the presence or absence of extrinsic material having the putative ability to activate the hormone responsive promoter/enhancer element,
(c) monitoring in the cells induction of the product of the reporter or desired DNA sequence, and
(d) measuring in the cells the expression of reporter or other desired DNA, the improvement wherein the receptor is one having increased trans-activation transcription activity compared with the parent receptor, the activity additional to the parent receptor by virtue of domains selected from the group comprising τ_1, τ_2, or functional fragments thereof, located within the molecule at a position outside of each of a DNA-binding and

ligand-binding domain of said receptor.

Claims for the following Contracting State : ES

1. An assay for screening and identifying materials having a putative potential of binding to a member of the steroid, thyroid, and retinoid superfamily of hormone receptors comprising the steps of:

   a) providing a member of the steroid hormone superfamily of receptors in a form having improved trans-activation transcription activity, wherein said receptor comprises at least one trans-activation transcription domain additional to the parent molecule selected from the group comprising τ_1, τ_2, and functional fragments thereof and located outside the DNA-binding and ligand-binding domain(s) of said receptor protein(s),
   (b) challenging the form of receptor species with one or more of a battery of test materials having putative potential of binding to a hormone or hormone-like receptor,
   (c) monitoring the effect of the test material by measuring the amount of transcription induced by the receptor species, and
   (d) selecting candidates from the battery of test materials capable of having an effect on the trans-activation transcription activity of said receptor species.

2. An assay as claimed in Claim 1 further comprising the additional step following step (d), comprising:
   (e) employing the candidate in the preparation of a composition containing the candidate as an essential component, the composition being useful to impart its biofunction properties on a corresponding receptor when it is contacted with the receptor.

3. An assay as claimed in Claim 2 further comprising the additional step following step (e), comprising:
   (f) contacting the composition with biological material obtainable from a human subject.

4. A method for the production of a member of the steroid, thyroid and retinoid hormone superfamily of receptors as a polypeptide having increased trans-activation transcription activity of a promoter with which it is associated, by virtue of its intrinsic ability to bind to a DNA sequence response element of the promoter or by its ability to associate with other polypeptides that bind to the DNA sequence response element, wherein the receptor contains at least one trans-activation transcription domain additional to the parent molecule selected from the group comprising τ_1, τ_2, and functional fragments thereof, located within the molecule in a location outside of its DNA-binding and ligand-binding domains, said method comprising transfecting a DNA encoding said receptor in a host cell and expressing said receptor.

5. A method as claimed in Claim 4 wherein the receptor is based on the human glucocorticoid receptor.

6. A method as claimed in Claim 4 wherein the trans-activation transcription domain is the τ_1 domain.

7. A method as claimed in Claim 6 wherein the receptor contains two of the τ_1 domains.

8. A method as claimed in Claim 7 wherein the second of the τ_1 domains is located adjacent to the first.

9. A method as claimed in Claim 7 wherein the second of the τ_1 domains is located in a C-terminal distal location from the first τ_1 sequence.

10. A method as claimed in any one of Claims 4 to 9 wherein said receptor lacks a ligand-binding domain.

11. A method as claimed in Claim 4 wherein a species of said receptor is human G442 glucocorticoid receptor, wherein by point mutation a glycine has replaced the native lysine at position 442.

12. A method for the production of a member of the steroid, thyroid and retinoid hormone superfamily of receptors having trans-activation transcription domains additional to that of the parent receptor selected from the group comprising τ_1, τ_2, and functional fragments thereof, autonomous with both the DNA-binding and ligand-binding domains of the parent receptor, the additional trans-activation transcription domains being located in a position outside of each of the DNA-binding and ligand-binding domains, if present, and having a DNA-binding biofunctionality, said method comprising transfecting a DNA encoding said receptor in a host cell and expressing said

receptor.

13. A recombinant method for the production of a DNA molecule that is a recombinant DNA molecule or a cDNA molecule encoding a receptor as claimed in Claim 12.

14. A recombinant method for the production of an expression vector operatively harboring DNA encoding a receptor as claimed in Claim 13.

15. A recombinant method for the production of a recombinant host cell transfected with an expression vector as claimed in Claim 14.

16. A method for the production of a cell culture comprising cells as claimed in claim 15 and an extrinsic support medium assuring the viability of the cells.

17. A process which comprises recovering and purifying a receptor as claimed in Claim 12 to a form having quality and quantity sufficient to enable its use in assays that enable measurement of extrinsically induced biofuctionality of the receptor.

18. In a bioassay for determining the functionality of a member of the steroid, thyroid and retinoid hormone superfamily of receptors, the bioassay comprising:

(a) transfecting into receptor negative cells one or more expression vectors harboring an operative hormone responsive promoter/enhancer element functionally linked to an operative reporter or other desired DNA and an operative DNA sequence encoding the receptor,
(b) culturing the transfected cells from step (a) in the presence or absence of extrinsic material having the putative ability to activate the hormone responsive promoter/enhancer element,
(c) monitoring in the cells induction of the product of the reporter or desired DNA sequence, and
(d) measuring in the cells the expression of reporter or other desired DNA, the improvement wherein the receptor is one having increased trans-activation transcription activity compared with the parent receptor, the activity additional to the parent receptor by virtue of domains selected from the group comprising τ_1, τ_2, or functional fragments thereof, located within the molecule at a position outside of each of a DNA-binding and ligand-binding domain of said receptor.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, LI, DE, FR, GB, GR, IT, LU, NL, SE

1. Test zum Screenen und Identifizieren von Materialien mit einem vermutlichen Potential zur Bindung an ein Mitglied der Steroid-, Thyroid- und der Retinoid-Superfamilie von Hormonrezeptoren umfassend die Schritte:

(a) Bereitstellen eines Mitglieds der Seroid-Hormon-Superfamilie von Rezeptoren in einer Form mit einer verbesserten Transkativierungs-Transkriptionsaktivität, worin der Rezeptor mindestens eine Transaktivierungs-Transkriptionsdomäne zusätzlich zum Ausgangsmolekül enthält, ausgewählt aus τ_1, τ_2 und funktionalen Fragmenten davon, und die außerhalb der DNA-Bindung und Ligand-Bindungsdomäne(n) des (der) Rezeptorprotein(e) lokalisiert ist,

(b) Exponieren der Form der Rezeptorspezies mit einer oder mehreren Reihen an Testmaterialien mit vermutetem Potential zur Bindung an ein Hormon oder Hormon-ähnlichen Rezeptor,

(c) Überwachen der Wirkung des Testmaterials durch Messen der durch die Rezeptorspezies induzierten Transkriptionsmenge und

(d) Auswählen von Kandidaten aus den Testreihenmaterialien, die eine Wirkung auf die Transaktivierungs-Transkriptionsaktivität der Rezeptorspezies haben können.

2. Test nach Anspruch 1, der ferner den zusätzlichen Schritt (d) umfaßt:

(e) Einsetzen des Kandidaten zur Herstellung einer Zusammensetzung, enthaltend den Kandidaten als wesentlichen Bestandteil, wobei die Zusammensetzung geeignet zur Verleihung von Biofunktionseigenschaften für einen entsprechenden Rezeptor ist, wenn sie mit dem Rezeptor in Kontakt gebracht wird.

3. Test nach Anspruch 2, der ferner den zusätzlichen Schritt (e) enthält:
(f) in Kontakt bringen der Zusammensetzung mit biologischem Material, erhältlich aus einem Menschen.

4. Mitglied der Steroid-, Thyroid- und Retinoid-Hormon-Superfamilie von Rezeptoren als ein Polypeptid mit erhöhter Transaktivierungs-Transkriptionsaktivität eines Promotors, mit dem dies verbunden ist, aufgrund seiner intrinsischen Aktivität an ein DNA-Sequenz-Response-Elements des Promotors oder durch seine Fähigkeit zur Assoziierung mit anderen Polypeptiden, die an das DNA-Sequenz-Response-Element binden, worin der Rezeptor mindestens eine Transaktivierungs-Transkriptionsdomäne zusätzlich zu dem Ausgangsmolekül enthält, ausgewählt aus τ_1, τ_2 und funktionellen Fragmenten davon, lokalisiert innerhalb des Moleküls an einem Ort außerhalb seiner DNA-Bindungs- und Ligand-Bindungs-Domänen.

5. Rezeptor nach Anspruch 4, worin der Rezeptor auf einem humanen Glucocorticoid-Rezeptor beruht.

6. Rezeptor nach Anspruch 4, worin die Transaktivierungs-Transkriptionsdomäne die τ_1-Domäne ist.

7. Rezeptor nach Anspruch 6, worin der Rezeptor zwei der τ_1-Domänen enthält.

8. Rezeptor nach Anspruch 7, worin die zweite der τ_1-Domänen zur ersten benachbart angeordnet ist.

9. Rezeptor nach Anspruch 7, worin die zweite der τ_1-Domänen distal zum C-Terminus entfernt von der ersten τ_1-Sequenz angeordnet ist.

10. Rezeptor nach Ansprüche 4 bis 9, dem eine Ligand-Bindungsdomäne fehlt.

11. Rezeptorspezies nach Anspruch 4, die der humane G442-Glucocorticoid-Rezeptor ist, worin durch Punkt-Mutation ein Glycin das native Lysin an Position 442 ersetzt hat.

12. Mitglied der Steroid-, Thyroid- und Retinoid-Hormon-Superfamilie von Rezeptoren mit Transaktivierungs-Transkriptionsdomänen zusätzlich zu dem Ausgangsrezeptor, ausgewählt aus τ_1, τ_2 und funktionalen Fragmenten davon, die autonom sowohl mit der DNA-Bindungs- und den Ligand-Bindungs-Domänen des Ausgangsrezeptors sind, wobei die zusätzlichen Transaktivierungs-Transkriptionsdomänen an einer Stelle außerhalb jeweils der DNA-Bindungs- und Ligand-Bindungs-Domänen, falls vorhanden, lokalisiert sind, und mit einer DNA-Bindungs-Bifunktionalität.

13. DNA-Molekül, welches ein rekombinantes DNA-Molekül oder ein cDNA-Molekül ist, das einen Rezeptor nach Anspruch 12 kodiert.

14. Expressionsvektor der operativ DNA enthält, welcher einen Rezeptor nach Anspruch 13 kodiert.

15. Rekombinante Wirtszelle transfiziert mit einem Expressionsvektor nach Anspruch 14.

16. Zellkultur, umfassend Zellen nach Anspruch 15 und ein extrinsisches Trägermedium, das die Lebensfähigkeit der Zellen sicherstellt.

17. Verfahren zur Herstellung eines humanen Rezeptors nach Anspruch 12, welches die Expression in einem rekombinanten Wirt die den Zell-transfizierende DNA-kodierenden Rezeptor umfaßt.

18. Verfahren, welches die Gewinnung und Reinigung eines Rezeptors nach Anspruch 12 in einer Form mit einer Qualität und Quantität umfaßt, die ausreichend zu seiner Befähigung bei der Verwendung in Tests ist, und die Messung von extrinsisch induzierter Biofunktionalität des Rezeptors ermöglicht.

19. Biotest zur Bestimmung der Funktionalität eines Mitglieds der Steroid-, Thyroid- und Retionoid-Hormon-Superfamilie von Rezeptoren, wobei der Biotest umfaßt:

(a) Transfektion von Rezeptor negative Zellen von einem oder mehreren Expressionsvektoren, die ein operatives Hormon-responsives Promotor/Enhancer-Element enthalten das funktional an einen operativen Reporter oder eine andere erwünschte DNA gebunden ist, und eine operative DNA-Sequenz kodierend den Rezeptor,

(b) Kultivieren der transfizierten Zellen aus Schritt (a) in Gegenwart oder Fehlen des extrinsischen Materials mit der vermuteten Fähigkeit zur Aktivierung des Hormon-responsiven Promotor/Enhancer-Elements,

(c) Überwachung der Zellinduktion des Produkts der Reporter oder erwünschten DNA-Sequenz, und

(d) Messen der Expression in den Zellen der Reporter oder der anderen erwünschten DNA, wobei die Verbesserung des Rezeptors eine mit erhöhter Transaktivierungs-Transkriptionsaktivität ist, im Vergleich zum Ausgangsrezeptor, wobei die Aktivität zusätzlich zum Ausgangsrezeptor aufgrund der Domänen, ausgewählt aus τ_1, τ_2 oder funktionalen Fragmenten davon, innerhalb des Moleküls an einer Position außerhalb einer jeden DNA-Bindungs- und Ligand-Bindungs-Domäne des Rezeptors lokalisiert ist.

Patentansprüche für folgenden Vertragsstaat : ES

1. Test zum Screenen und Identifizieren von Materialien mit einem vermutlichen Potential zur Bindung an ein Mitglied der Steroid-, Thyroid- und der Retinoid-Superfamilie von Hormonrezeptoren umfassend die Schritte:

(a) Bereitstellen eines Mitglieds der Seroid-Hormon-Superfamilie von Rezeptoren in einer Form mit einer verbesserten Transkativierungs-Transkriptionsaktivität, worin der Rezeptor mindestens eine Transaktivierungs-Transkriptionsdomäne zusätzlich zum Ausgangsmolekül enthält, ausgewählt aus τ_1, τ_2 und funktionalen Fragmenten davon, und die außerhalb der DNA-Bindung und Ligand-Bindungsdomäne(n) des (der) Rezeptorprotein(e) lokalisiert ist,

(b) Exponieren der Form der Rezeptorspezies mit einer oder mehreren Reihen an Testmaterialien mit vermutetem Potential zur Bindung an ein Hormon oder Hormon-ähnlichen Rezeptor,

(c) Überwachen der Wirkung des Testmaterials durch Messen der durch die Rezeptorspezies induzierten Transkriptionsmenge und

(d) Auswählen von Kandidaten aus den Testreihenmaterialien, die eine Wirkung auf die Transaktivierungs-Transkriptionsaktivität der Rezeptorspezies haben können.

2. Test nach Anspruch 1, der ferner den zusätzlichen Schritt (d) umfaßt:
(e) Einsetzen des Kandidaten zur Herstellung einer Zusammensetzung enthaltend den Kandidaten als wesentlichen Bestandteil, wobei die Zusammensetzung geeignet zur Verleihung von Biofunktionseigenschaften für einen entsprechenden Rezeptor ist, wenn sie mit dem Rezeptor in Kontakt gebracht wird.

3. Test nach Anspruch 2, der ferner den zusätzlichen Schritt (e) enthält:
(f) in Kontakt bringen der Zusammensetzung mit biologischem Material, erhältlich aus einem Menschen.

4. Verfahren zur Produktion eines Mitglieds der Steroid-, Thyroid- und Retinoid-Hormon-Superfamilie von Rezeptoren als ein Polypeptid mit erhöhter Transaktivierungs-Transkriptionsaktivität eines Promotors, mit dem dies verbunden ist, aufgrund seiner intrinsischen Aktivität an ein DNA-Sequenz-Response-Elements des Promotors oder durch seine Fähigkeit zur Assoziierung mit anderen Polypeptiden, die an das DNA-Sequenz-Response-Element binden, worin der Rezeptor mindestens eine Transaktivierungs-Transkriptionsdomäne zusätzlich zu dem Ausgangsmolekül enthält, ausgewählt aus τ_1, τ_2 und funktionellen Fragmenten davon, lokalisiert innerhalb des Moleküls an einem Ort außerhalb seiner DNA-Bindungs- und Ligand-Bindungs-Domänen, worin das Verfahren die Transfektion einer DNA, die diesen Rezeptor kodiert, in eine Wirtszelle und Expression des Rezeptors umfaßt.

5. Verfahren nach Anspruch 4, worin der Rezeptor auf einem humanen Glucocorticoid-Rezeptor beruht.

6. Verfahren nach Anspruch 4, worin die Transaktivierungs-Transkriptionsdomäne die τ_1-Domäne ist.

7. Verfahren nach Anspruch 6, worin der Rezeptor zwei τ_1-Domänen enthält.

8. Verfahren nach Anspruch 7, worin die zweite der τ_1-Domänen zur ersten benachbart angeordnet ist.

9. Verfahren nach Anspruch 7, worin die zweite der τ_1-Domänen distal zum C-Terminus entfernt von der ersten τ_1-Sequenz angeordnet ist.

10. Verfahren nach Ansprüche 4 bis 9, dem eine Ligand-Bindungsdomäne fehlt.

11. Verfahren nach Anspruch 4, die der humane G442-Glucocorticoid-Rezeptor ist, worin durch Punkt-Mutation ein Glycin das native Lysin an Position 442 ersetzt hat.

12. Verfahren zur Herstellung eines Mitglieds der Steroid-, Thyroid- und Retinoid-Hormon-Superfamilie an Rezeptoren mit Transaktivierungs-Transkriptionsdomänen zusätzlich zu dem Ausgangsrezeptor, ausgewählt aus τ_1, τ_2 und funktionalen Fragmenten davon, die autonom sowohl mit der DNA-Bindungs- und den Ligand-Bindungs-Domänen des Ausgangsrezeptors sind, wobei die zusätzlichen Transaktivierungs-Transkriptionsdomänen an einer Stelle außerhalb jeweils der DNA-Bindungs- und Ligand-Bindungs-Domänen, falls vorhanden, lokalisiert sind und mit einer DNA-Bindungs-Bifunktionalität, worin das Verfahren die Transfektion einer DNA, die diesen Rezeptor kodiert, in eine Wirtszelle und Expression des Rezeptors umfaßt.

13. Rekombinantes Verfahren zur Herstellung eines DNA-Moleküls, das ein rekombinantes DNA-Molekül oder ein cDNA-Molekül ist, das den Rezeptor nach Anspruch 12 kodiert.

14. Rekombinantes Verfahren zur Herstellung eines Expressionsvektors der operativ DNA enthält, welcher einen Rezeptor nach Anspruch 13 kodiert.

15. Rekombinantes Verfahren zur Herstellung einer rekombinanten Wirtszelle, transfiziert mit einem Expressionsvektor nach Anspruch 14.

16. Verfahren zur Herstellung einer Zellkultur, umfassend Zellen nach Anspruch 15 und ein extrinsisches Trägermedium, das die Lebensfähigkeit der Zellen sicherstellt.

17. Verfahren, das die Wiedergewinnung und Reinigung eines Rezeptors nach Anspruch 12 umfaßt, in einer Form mit einer Qualität und Quantität, die ausreichend zur Sicherstellung seiner Verwendung in Tests ist, die die Messung von extrinsisch induzierten Biofunktionalität des Rezeptors ermöglichen.

18. Biotest zur Bestimmung der Funktionalität eines Mitglieds der Steroid-, Thyroid- und Retionoid-Hormon-Superfamilie von Rezeptoren, wobei der Biotest umfaßt:

(a) Transfektion von Rezeptor negative Zellen von einem oder mehreren Expressionsvektoren, die ein operatives Hormon-responsives Promotor/Enhancer-Element enthalten das funktional an einen operativen Reporter oder eine andere erwünschte DNA gebunden ist, und eine operative DNA-Sequenz kodierend den Rezeptor,

(b) Kultivieren der transfizierten Zellen aus Schritt (a) in Gegenwart oder Fehlen des extrinsischen Materials mit der vermuteten Fähigkeit zur Aktivierung des Hormon-responsiven Promotor/Enhancer-Elements,

(c) Überwachung der Zellinduktion des Produkts der Reporter oder erwünschten DNA-Sequenz, und

(d) Messen der Expression in den Zellen der Reporter oder der anderen erwünschten DNA, wobei die Verbesserung des Rezeptors eine mit erhöhter Transaktivierungs-Transkriptionsaktivität ist, im Vergleich zum Ausgangsrezeptor, wobei die Aktivität zusätzlich zum Ausgangsrezeptor aufgrund der Domänen, ausgewählt aus τ_1, τ_2 oder funktionalen Fragmenten davon, innerhalb des Moleküls an einer Position außerhalb einer jeden DNA-Bindungs- und Ligand-Bindungs-Domäne des Rezeptors lokalisiert ist.

Revendications

Revendications pour les Etats contractants uivants : AT, BE, CH, LI, DE, FR, GB, GR, IT, LU, NL, SE

1. Essai de sélection et d'identification de substances ayant une capacité potentielle à se lier à un membre de la superfamille des récepteurs des hormones stéroïdes, thyroïdiennes et rétinoïdes comprenant les étapes suivantes consistant :

   a) à fournir un membre de la superfamille des récepteurs des hormones stéroïdes sous une forme ayant une meilleure activité de *trans*-activation de la transcription, ledit récepteur comprenant au moins un domaine de *trans*-activation de la transcription en plus que la molécule d'origine, choisi dans le groupe comprenant τ_1, τ_2 et des fragments fonctionnels de ceux-ci, et situés en dehors du ou des domaines de liaison à l'ADN et au ligand dudit ou desdits récepteurs protéiques,
   b) à mettre l'espèce de récepteur en contact avec une ou plusieurs d'une série de substances à tester ayant une capacité potentielle à se lier à un récepteur d'hormones ou de molécules de type hormone,
   (c) à suivre l'effet de la substance à tester en mesurant le niveau de transcription induite par le récepteur, et
   (d) à sélectionner des substances candidates dans une série de substances à tester capables d'avoir un effet sur l'activité de *trans*-activation de la transcription dudit récepteur.

2. Essai conforme à la revendication 1, comprenant en outre une étape supplémentaire, suivant l'étape (d), comprenant :
   (e) l'utilisation de la substance candidate pour la préparation d'une composition contenant la substance candidate comme un composant essentiel, la composition étant utile pour conférer au récepteur correspondant ses propriétés biofonctionnelles lorsqu'elle est mise en contact avec le récepteur.

3. Essai conforme à la revendication 2, comprenant en outre une étape supplémentaire, suivant l'étape (e), consistant
   (f) à mettre en contact la composition avec des substances biologiques pouvant être obtenues à partir d'un être humain.

4. Membre de la superfamille des récepteurs des hormones stéroïdes, thyroïdiennes et rétinoïdes sous forme d'un polypeptide ayant une activité accrue de *trans*-activation de la transcription d'un promoteur auquel il est associé, grâce à sa capacité intrinsèque à se lier à une séquence d'ADN sensible aux récepteurs hormonaux du promoteur ou grâce à sa capacité à s'associer à d'autres polypeptides qui se lient à une séquence d'ADN sensible aux récepteurs hormonaux, le récepteur contenant au moins un domaine de *trans*-activation de la transcription de plus que la molécule d'origine, choisi dans le groupe comprenant τ_1, τ_2 et des fragments fonctionnels de ceux-ci, situé dans la molécule à un endroit en dehors des domaines de liaison à l'ADN et de liaison au ligand.

5. Récepteur conforme à la revendication 4 dans lequel le récepteur est basé sur le récepteur humain des glucocorticoïdes.

6. Récepteur conforme à la revendication 4 dans lequel le domaine de *trans*-activation de la transcription est le domaine τ_1.

7. Récepteur conforme à la revendication 6 dans lequel le récepteur contient deux domaines τ_1.

8. Récepteur conforme à la revendication 7 dans lequel le second domaine τ_1 est en position adjacente au premier.

9. Récepteur conforme à la revendication 7 dans lequel le second domaine τ_1 est situé en position C-terminale au delà de la première séquence τ_1.

10. Récepteur conforme à une quelconque des revendications 4 à 9 ne possédant pas de domaine de liaison au ligand.

11. Espèce d'un récepteur conforme à la revendication 4 qui est le récepteur humain des glucocorticoïdes G442 dans lequel la lysine native en pqsition 442 a été remplacée par une glycine par mutation ponctuelle.

12. Membre de la superfamille des récepteurs des hormones stéroïdiennes, thyroïdiennes et rétinoïdes comportant un ou plusieurs domaines de *trans*-activation de la transcription de plus que le récepteur d'origine, lequel domaine

est choisi dans le groupe formé par τ_1, τ_2 et les fragments fonctionnels de ceux-ci, lesquels domaines sont autonomes vis-à-vis des domaines de liaison à l'ADN et de liaison au ligand du récepteur d'origine, les domaines supplémentaires de *trans*-activation de la transcription étant situés en dehors de chacun des domaines de liaison à l'ADN et au ligand, s'ils sont présents, et ayant une fonction biologique de liaison à l'ADN.

13. Molécule d'ADN qui est une molécule d'ADN recombiné ou une molécule d'ADNc codant un récepteur conforme à la revendication 12.

14. Vecteur d'expression renfermant de manière opérationnelle de l'ADN codant un récepteur conforme à la revendication 13.

15. Cellule hôte de recombinaison transformée par un vecteur d'expression conforme à la revendication 14.

16. Culture de cellules constituée de cellules conformes à la revendication 15 et d'un milieu de support extrinsèque assurant la viabilité des cellules.

17. Procédé de préparation d'un récepteur humain conforme à la revendication 12 comprenant l'expression, dans une cellule hôte de recombinaison, d'un ADN transformant codant un récepteur.

18. Procédé comprenant la récupération et la purification d'un récepteur conforme à la revendication 12 afin d'obtenir un récepteur en une quantité et avec une qualité permettant son utilisation dans des essais permettant la mesure de la fonction biologique du récepteur induite par voie extrinsèque.

19. Essai biologique pour la détermination de la fonction d'un membre de la superfamille des récepteurs des hormones stéroïdiennes, thyroïdiennes et rétinoïdes, l'essai biologique comprenant :

(a) la transfection, dans des cellules récepteur-négatives, d'un ou de plusieurs vecteurs d'expression renfermant un élément promoteur/amplificateur opérationnel sensible aux hormones, lié fonctionnellement à un gène rapporteur opérationnel ou à un autre ADN choisi, et une séquence d'ADN opérationnelle codant le récepteur,
(b) la culture des cellules transformées obtenues dans l'étape (a) en présence ou en absence de substances extrinsèques ayant potentiellement la capacité à activer l'élément promoteur/amplificateur sensible aux récepteurs hormonaux,
(c) le fait de suivre dans les cellules, l'induction de l'expression du gène rapporteur ou de la séquence d'ADN choisie, et
(d) la mesure, dans les cellules, de l'expression de l'ADN rapporteur ou d'un autre ADN choisi, dans lequel l'amélioration réside dans un récepteur ayant une activité accrue de la *trans*-activation de la transcription par rapport à celle du récepteur d'origine, l'augmentation de l'activité par rapport au récepteur d'origine étant due à la présence de domaines choisis dans le groupe formé de τ_1, τ_2 ou de fragments fonctionnels de ceux-ci, situés dans la molécule en une position en dehors du domaine de liaison à l'ADN et du domaine de liaison au ligand dudit récepteur.

Revendications pour l'Etat contractant suivant : ES

1. Essai de sélection et d'identification de substances ayant une capacité potentielle à se lier à un membre de la superfamille des récepteurs des hormones stéroïdes, thyroïdiennes et rétinoïdes comprenant les étapes suivantes consistant :

a) à fournir un membre de la superfamille des récepteurs des hormones stéroïdes sous une forme ayant une meilleure activité de *trans*-activation de la transcription, ledit récepteur comprenant au moins un domaine de *trans*-activation de la transcription en plus que la molécule d'origine, choisi dans le groupe comprenant τ_1,τ_2 et des fragments fonctionnels de ceux-ci, et situés en dehors du ou des domaines de liaison à l'ADN et au ligand dudit ou desdits récepteurs protéiques,
b) à mettre l'espèce de récepteur en contact avec une ou plusieurs d'une série de substances à tester ayant une capacité potentielle à se lier à un récepteur d'hormones ou de molécules de type hormone,
(c) à suivre l'effet de la substance à tester en mesurant le niveau de transcription induite par le récepteur, et
(d) à sélectionner des substances candidates dans une série de substances à tester capables d'avoir un effet

sur l'activité de *trans*-activation de la transcription dudit récepteur.

2. Essai conforme à la revendication 1, comprenant en outre une étape supplémentaire, suivant l'étape (d), comprenant :
(e) l'utilisation de la substance candidate pour la préparation d'une composition contenant la substance candidate comme un composant essentiel, la composition étant utile pour conférer au récepteur correspondant ses propriétés biofonctionnelles lorsqu'elle est mise en contact avec le récepteur.

3. Essai conforme à la revendication 2, comprenant en outre une étape supplémentaire, suivant l'étape (e), consistant (f) à mettre en contact la composition avec des substances biologiques pouvant être obtenues à partir d'un être humain.

4. Procédé de préparation d'un membre de la superfamille des récepteurs des hormones stéroïdes, thyroïdiennes et rétinoïdes sous forme d'un polypeptide ayant une activité accrue de *trans*-activation de la transcription d'un promoteur auquel il est associé, grâce à sa capacité intrinsèque à se lier à une séquence d'ADN sensible aux récepteurs hormonaux du promoteur ou grâce à sa capacité à s'associer à d'autres polypeptides qui se lient à une séquence d'ADN sensible aux récepteurs hormonaux, le récepteur contenant au moins un domaine de *trans*-activation de la transcription de plus que la molécule d'origine, choisi dans le groupe comprenant τ_1, τ_2 et des fragments fonctionnels de ceux-ci, situé dans la molécule à un endroit en dehors des domaines de liaison à l'ADN et de liaison au ligand, ledit procédé comprenant la transfection d'un ADN codant ledit récepteur dans une cellule hôte et l'expression dudit récepteur.

5. Procédé conforme à la revendication 4 dans lequel le récepteur est basé sur le récepteur humain des glucocorticoïdes.

6. Procédé conforme à la revendication 4 dans lequel le domaine de *trans*-activation de la transcription est le domaine τ_1.

7. Procédé conforme à la revendication 6 dans lequel le récepteur contient deux domaines τ_1.

8. Procédé conforme à la revendication 7 dans lequel le second domaine τ_1 est en position adjacente au premier.

9. Procédé conforme à la revendication 7 dans lequel le second domaine τ_1 est situé en position C-terminale au delà de la première séquence τ_1.

10. Procédé conforme à une quelconque des revendications 4 à 9 dans lequel ledit récepteur ne possède pas de domaine de liaison au ligand.

11. Procédé conforme à la revendication 4 dans lequel une espèce dudit récepteur est le récepteur humain des glucocorticoïdes G442 dans lequel la lysine native en position 442 a été remplacée par une glycine par mutation ponctuelle.

12. Procédé de prépartion d'un membre de la superfamille des récepteurs des hormones stéroïdiennes, thyroïdiennes et rétinoïdes comportant un ou plusieurs domaines de *trans*-activation de la transcription de plus que le récepteur d'origine, lequel domaine est choisi dans le groupe formé par τ_1, τ_2 et les fragments fonctionnels de ceux-ci, lesquels domaines sont autonomes vis-à-vis des domaines de liaison à l'ADN et de liaison au ligand du récepteur d'origine, les domaines supplémentaires de *trans*-activation de la transcription étant situés en dehors de chacun des domaines de liaison à l'ADN et au ligand, s'ils sont présents, et ayant une fonction biologique de liaison à l'ADN, ledit procédé comprenant la transfection d'un ADN codant ledit récepteur dans une cellule hôte et l'expression dudit récepteur.

13. Procédé de recombinaison pour la préparation d'une molécule d'ADN qui est une molécule d'ADN recombiné ou une molécule d'ADNc codant un récepteur conforme à la revendication 12.

14. Procédé de recombinaison pour la préparation d'un vecteur d'expression renfermant de manière opérationnelle de l'ADN codant un récepteur conforme à la revendication 13.

15. Procédé de recombinaison pour la préparation d'une cellule hôte de recombinaison transformée par un vecteur

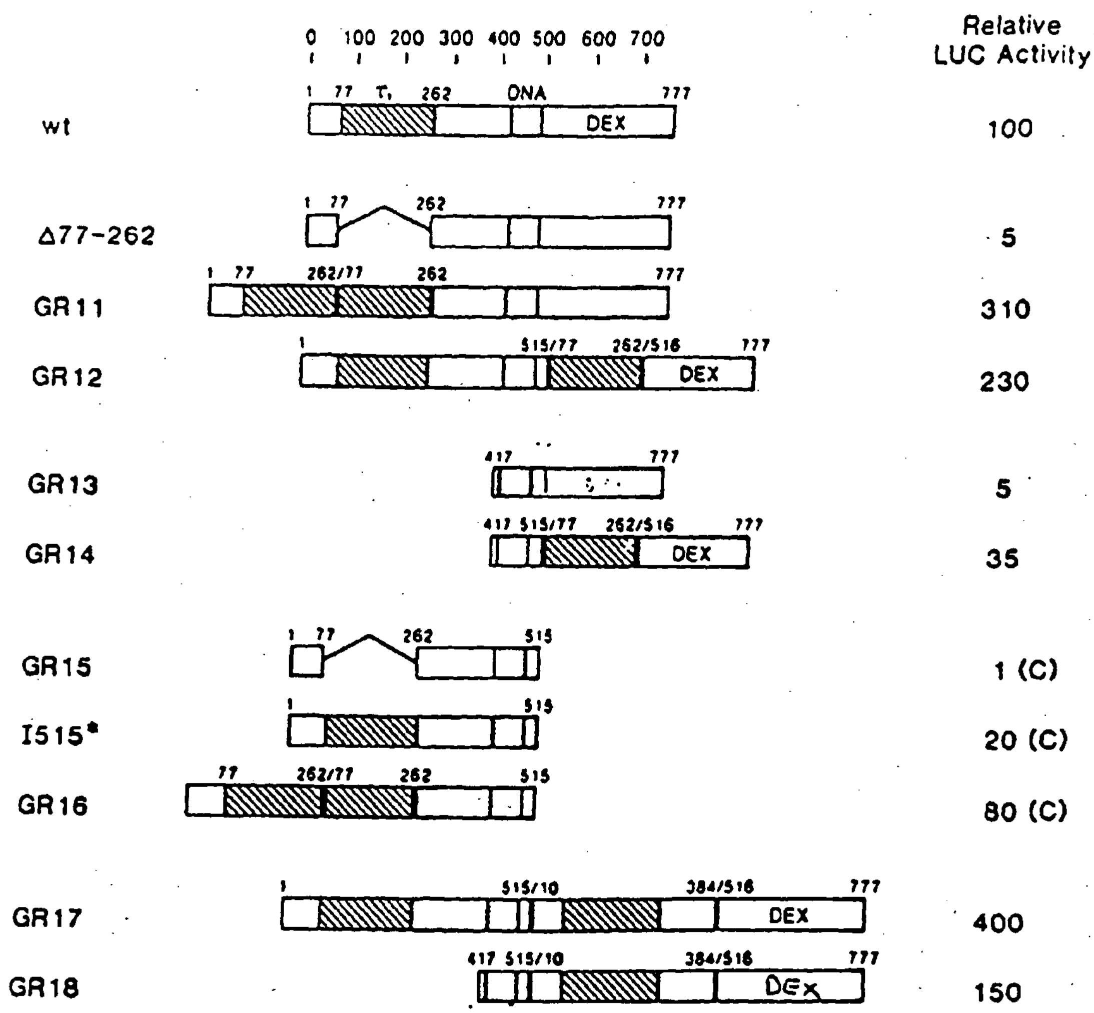
d'expression conforme à la revendication 14.

16. Procédé de préparation d'une culture de cellules constituée de cellules conformes à la revendication 15 et d'un milieu de support extrinsèque assurant la viabilité des cellules.

17. Procédé comprenant la récupération et la purification d'un récepteur conforme à la revendication 12 permettant d'obtenir un récepteur en une quantité et avec une qualité permettant son utilisation dans des essais destinés à la mesure de la fonction biologique du récepteur induite par voie extrinsèque.

18. Essai biologique pour la détermination de la fonction d'un membre de la superfamille des récepteurs des hormones stéroïdiennes, thyroïdiennes et rétinoïdes, l'essai biologique comprenant :

(a) la transfection, dans des cellules récepteur-négatives, d'un ou de plusieurs vecteurs d'expression renfermant un élément promoteur/amplificateur opérationnel sensible aux hormones, lié fonctionnellement à un gène rapporteur opérationnel ou à un autre ADN choisi, et une séquence d'ADN opérationnelle codant le récepteur,
(b) la culture des cellules transformées obtenues dans l'étape (a) en présence ou en absence de substances extrinsèques ayant potentiellement la capacité à activer l'élément promoteur/amplificateur sensible aux récepteurs hormonaux,
(c) le fait de suivre dans les cellules, l'induction de l'expression du gène rapporteur ou de la séquence d'ADN choisie, et
(d) la mesure, dans les cellules, de l'expression de l'ADN rapporteur ou d'un autre ADN choisi, dans lequel l'amélioration réside dans un récepteur ayant une activité accrue de la *trans*-activation de la transcription par rapport à celle du récepteur d'origine, l'augmentation de l'activité par rapport au récepteur d'origine étant due à la présence de domaines choisis dans le groupe formé de τ_1, τ_2 ou de fragments fonctionnels de ceux-ci, situés dans la molécule en une position en dehors du domaine de liaison à l'ADN et du domaine de liaison au ligand dudit récepteur.



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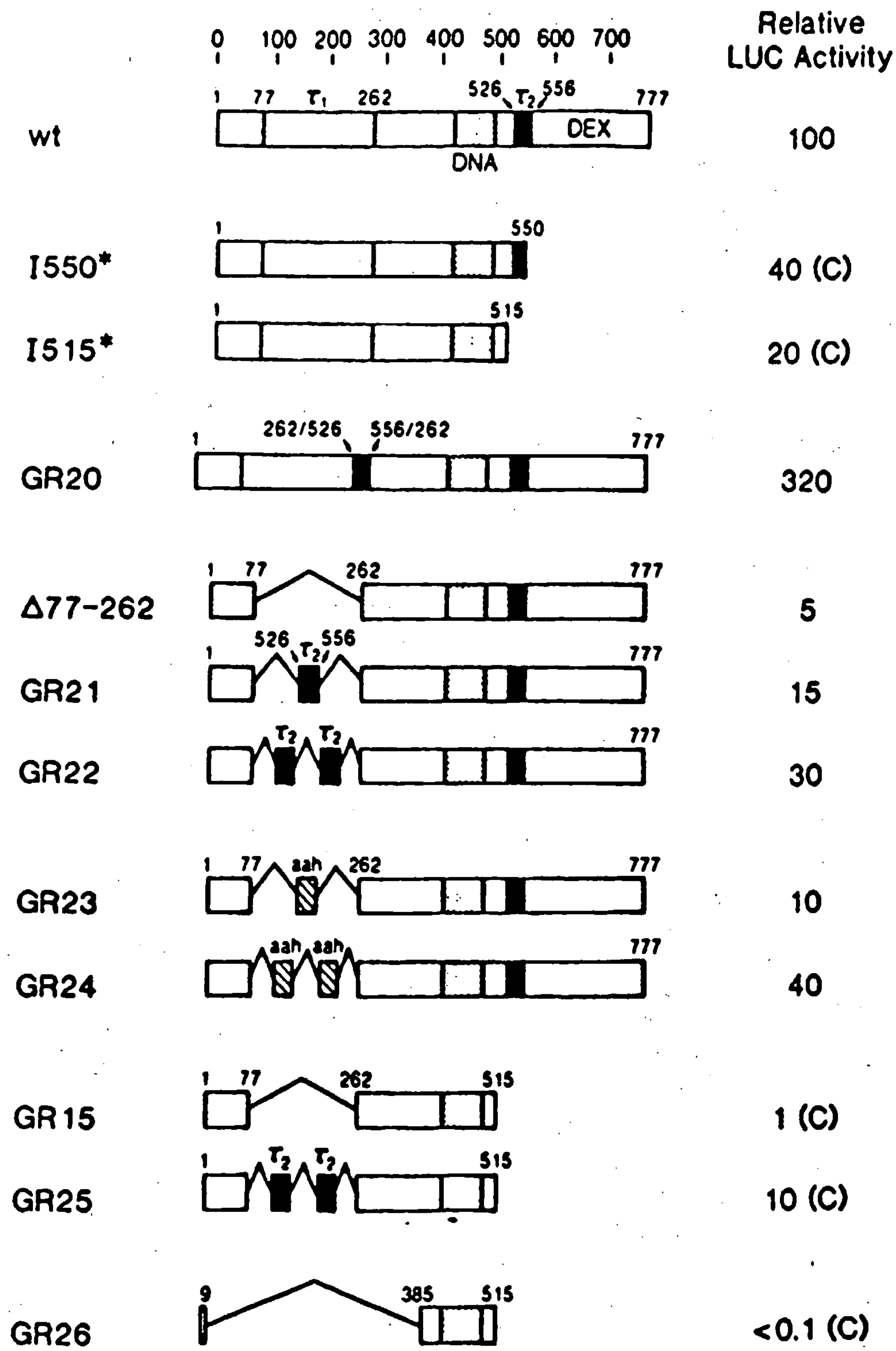
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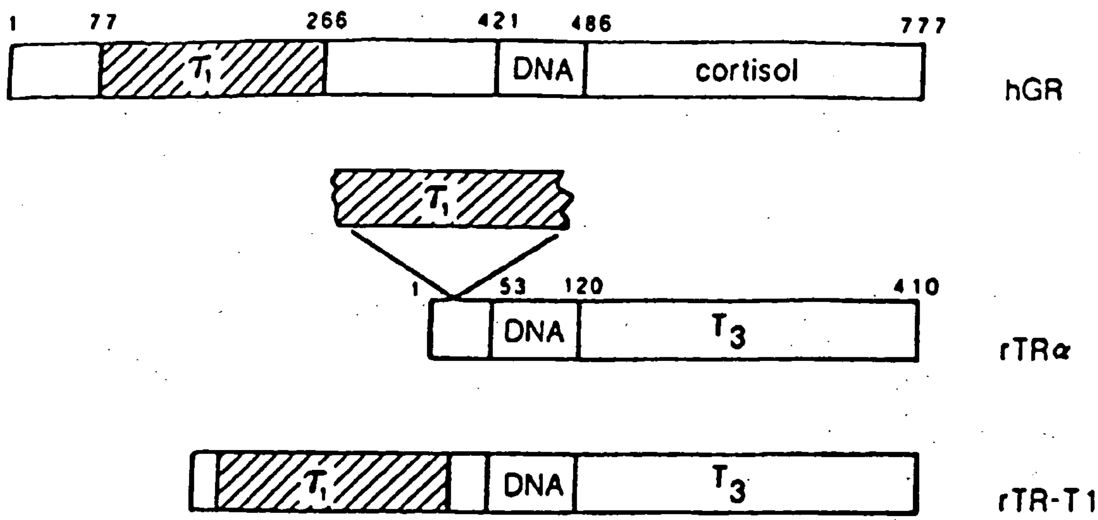
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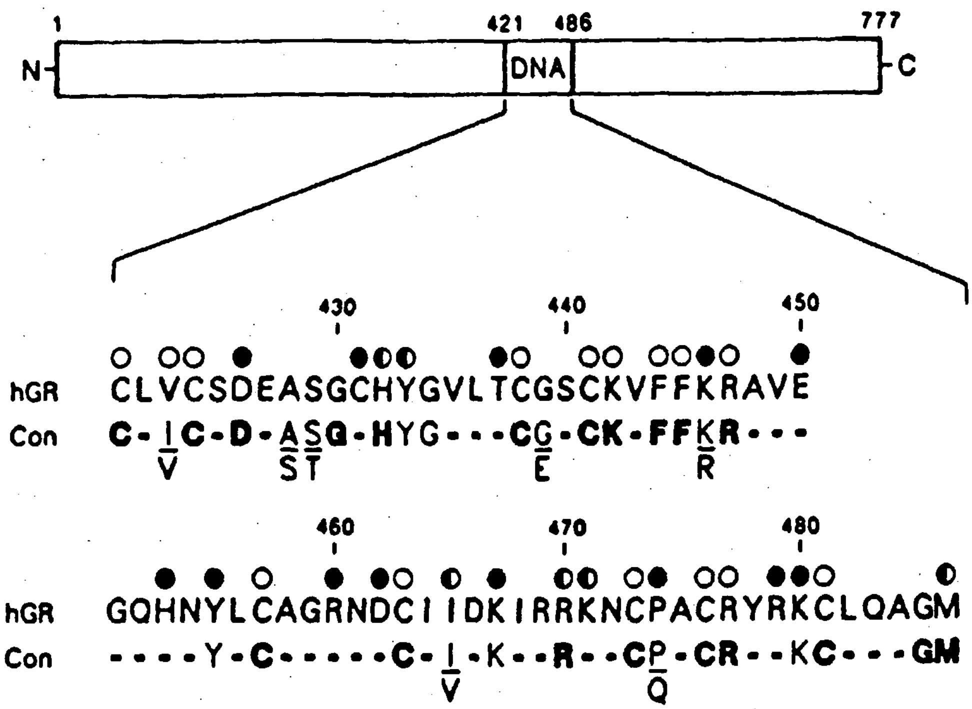
T1
DNA
T3
rTR-T1


1
421
486
777
N
DNA
C
430
440
450
hGR CLVCSDEASGCHYGVLTCGSCKVFFKRAVE
Con C-IC-D-ASG-HYG---CG-CK-FFKR---
V ST E R
460
470
480
hGR GQHNYLCAGRNDCIIDKIRRKNCPACRYRKCLQAGM
Con ----Y-C-----C-I-K--R--CP-CR--KC---GM
V Q